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Onset of Apoprotein E Secretion during Differentiation of Mouse Bone Marrow-derived Mononuclear Phagocytes

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ABSTRACT A number of macrophage functions were sequentially expressed when the bone marrow precursors of mononuclear phagocytes differentiated in culture in the presence of a specific growth factor, colony-stimulating factor-1. We have defined the expression of apoprotein E (ApoE), a major secreted protein of resident peritoneal macrophages, during maturation of adherent bone marrow-derived mononuclear phagocytes into macrophages. By 5 d the bone marrow macrophages were active secretory cells, but few cells contained intracellular immunoreactive ApoE, and little, if any, ApoE was secreted. ApoE secretion was initiated at 9 d, and this correlated with an increase in the percentage of macrophages containing intracellular ApoE. The onset of ApoE secretion was selective, and little change occurred in the other major secreted proteins detected by [^{35}S]methionine incorporation. In parallel, the high rate of plasminogen activator secretion, which peaked at 7 d, decreased markedly. ApoE secretion was not associated with altered expression of the macrophage surface antigen, Ia, or with secretion of fibronectin. Virtually all cells in independent colonies of bone marrow-derived macrophages eventually expressed ApoE. The proliferating monocyte/macrophage-like cell lines P388D1, J774.2, WEHI-3, RAW 264.1, and MGI.D$^+$ secreted little or no ApoE. These data establish that ApoE secretion is developmentally regulated.

The lineage-specific growth factor, colony-stimulating factor-1 (CSF-1[1]), induces hematopoietic stem cells to proliferate and differentiate into cells of the mononuclear phagocyte family (1, 2). When bone marrow cells are cultured with CSF-1 the mononuclear phagocytes proliferate and sequentially express some of the phenotypes and functions of the monocyte-macrophage lineage, including surface antigens (3), plasminogen activator (4, 5), response to migration inhibitory factor (6), lysozyme secretion (7), myeloperoxidase (6, 8, 9), Fc receptors (3, 6), and C3b receptors (10). Mature macrophages, such as those from peritoneal exudates, exhibit less extensive proliferation in the presence of CSF-1 (1, 4, 11), and resident peritoneal macrophages, although unable to proliferate, have receptors for CSF-1 (1, 12) and respond to it by increasing production of plasminogen activator (13).

During culture of bone marrow in the presence of CSF-1, cells become adherent by 2–3 d and sequentially express monoblast, promonocyte, monocyte, and macrophage morphology (1–6, 11). By 5 d, >90% of the adherent cells are macrophages (1), which are collectively called bone marrow-derived macrophages (BMM). During differentiation, bone marrow-derived macrophages transiently express enzymes and surface antigenic properties resembling those of immunologically activated macrophages (3, 6, 13, 14). It has been suggested that some of the functional heterogeneity of tissue macrophages arises as a result of the phenotypic expression of functions during differentiation (3, 14). We have recently determined that the expression of apoprotein E (ApoE), a component of plasma lipoproteins that is synthesized by macrophages (15), is regulated by the functional state of mature macrophages (16, 17). ApoE is synthesized and secreted by resident and thioglycollate-elicited macrophages, but not by immunologically activated macrophages (16, 17). Macrophages are active secretory cells, but, except for lysozyme (7) and plasminogen activator (4, 5), little is known about the regulation of specific secretory products during their differentiation from bone marrow precursors. Accordingly, we have studied the regulation of ApoE synthesis and secretion in bone marrow cells of the macrophage lineage differentiating in the presence of CSF-1.

[1] *Abbreviations used in this paper:* ApoE, apoprotein E; BCG, bacillus Calmette Guérin; BMM, bone marrow-derived macrophages; CSF-1, colony-stimulating factor-1.

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MATERIALS AND METHODS

Culture of Adherent Bone Marrow Macrophages: Bone marrow cells flushed from the femurs of female C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 10% horse serum (GIBCO, Grand Island, NY), and 10% L-cell-conditioned medium as the source of CSF-1, at $1-3 \times 10^4$ per 4-cm^2 culture well (Falcon Labware, Oxnard, CA), and cultured for 3–22 d as described previously (3, 4, 11). Because endotoxin, a possible contaminant of culture medium, suppresses ApoE secretion (16, 17), and has other effects on BMM (1, 19, 20), C3H/HeJ (endotoxin-resistant) mice were used in most experiments. Bone marrow cells initially plated at 1×10^4 cells per well grew into discrete colonies of mononuclear phagocytes by 3–7 d, giving rise to a confluent monolayer ($\sim 10^6$ cells) by 8–10 d. The resulting adherent populations were 100% macrophages by 5 d, as determined by morphology and esterase-staining (3, 6). Resident and elicited macrophages were obtained and cultured as described previously (11, 16). To study proliferating mature macrophages, peritoneal macrophages elicited by thioglycollate broth, $NaIO_4$, or bacillus Calmette Guérin (BCG) (16) were plated at 1×10^4 per 4-cm^2 culture well as described for BMM (11).

In experiments where indicated, peritoneal macrophages and BMM were incubated in culture with 5 μg/ml acetylated low density lipoproteins as described previously (15) for 24 h before analyzing secreted proteins to test the effect of cholesterol loading on ApoE secretion. Cholesterol loading was assessed by the appearance of intracellular lipid droplets.

Macrophage-like cell lines P388D1 (21), WEHI-3, and RAW 264.1 (22) were kindly provided by Dr. Peter Ralph (Sloan-Kettering Institute for Cancer Research, Rye, NY). J774.2 (23) was the gift of Dr. Jay C. Unkeless (Rockefeller University, New York, NY). MGI.D$^+$, clone 11 (24), was the gift of Dr. Leo Sachs (Weizmann Institute, Rehovot, Israel). J774 × RAM-1 (J774.2-rat alveolar macrophage hybrid) was the gift of Dr. Jerry Kaplan (University of Utah, Salt Lake City, UT). All cell lines were cultured in Dulbecco's medium supplemented with 10% fetal bovine serum.

Assays for Cellular and Secreted Proteins and Surface Antigens of Macrophages: IgG from a rabbit anti-rat ApoE serum (gift of Dr. Thomas Innerarity, Gladstone Foundation Laboratories, University of California, San Francisco) purified by affinity chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was characterized as specific after analysis of mouse serum, macrophage lysates, and macrophage-conditioned medium by double diffusion in agarose gels, by immunoprecipitation of a single species of protein from ^{125}I-labeled mouse very-low density lipoproteins and [^{35}S]methionine-labeled macrophages, by peptide mapping after partial proteolysis in gels of immunoprecipitated proteins from macrophages and very-low density lipoproteins, and by Western immunoblot analysis of mouse serum proteins and macrophages as documented previously (16, 17). Methods for radiolabeling macrophages with [^{35}S]methionine and analysis of cellular and secreted proteins by trichloracetic acid precipitation, 7–18% gradient SDS PAGE immunoprecipitation of ApoE using IgG from a specific rabbit anti-rat ApoE antiserum, and indirect immunofluorescent localization of ApoE using rhodamine-labeled IgG from sheep anti-rabbit IgG (Cappel Laboratories, West Chester, PA) were described previously (16, 17). In all the experiments described, macrophages were incubated for 2 h in the presence of 25 μCi/ml of [^{35}S]methionine, which has been shown to be optimal for observing cellular and secreted ApoE (16). ApoE secretion was quantified by immunoprecipitation or by densitometry of fluorograms (16). Assays for plasminogen activator and elastase were performed as described previously (25). Neutral proteinase was determined as plasminogen-independent lysis of ^{125}I-fibrin (25). 1 U of plasminogen activator, neutral proteinase, or elastase activity is defined as 1 μg of substrate degraded in 1 h at 37°C. Monoclonal antibody to Ia^k (I-A^k, specificity 17), expressed by C3H mice, was prepared by affinity chromatography of culture supernatants of hybridoma 10-2.16 (26) (Salk Cell Culture Center) on protein A-Sepharose. Distribution of this surface antigen was determined by indirect immunofluorescence using 1 μg/ml of the monoclonal antibody followed by fluorescein-labeled IgG from rabbit anti-mouse IgG (Cappel Laboratories) as second antibody. Actin (M_r 42,000) and lysozyme (M_r 14,000) were identified by isoelectric point and electrophoretic mobility in one- and two-dimensional polyacrylamide gels (16). Fibronectin secreted by macrophages was identified by its binding affinity for *Staphylococcus aureus* (27) and for gelatin-agarose (28), as described previously (16).

RESULTS

Appearance of ApoE Secretion during Culture of Bone Marrow-derived Macrophages

BMM were active in biosynthesis and secretion of proteins (Table I). By 5 d many secreted proteins characteristic of macrophages (e.g., lysozyme, the major band at M_r 14,000) were evident, but immunoprecipitable ApoE did not appear until 9 d (Fig. 1*a*). Except for the appearance of ApoE and the disappearance of a polypeptide (M_r 30,000) representing ~2% of the total, the major secreted proteins changed little between 5 and 12 d. Intracellular ApoE, determined by immunoprecipitation (Fig. 1*b*) and by immunofluorescence (Fig. 2), was also first seen at 9 d of culture. Overall patterns of cell-associated proteins synthesized by BMM changed markedly between 7 and 9 d in culture. Notably, synthesis of a cellular protein of M_r 28,000 decreased and synthesis of actin (M_r 42,000) increased at this time. The increase in ApoE secretion, as a percentage of total secretion, slightly lagged behind the increase in the percentage of macrophages containing intracellular immunofluorescent ApoE (Table I, Fig. 3). However, the intracellular ApoE, which was first seen as a

TABLE I

Incorporation of [^{35}S]Methionine into Proteins of BMM

Days in culture	[^{35}S]methionine incorporated*		ApoE‡	
	Cellular and secreted	Secreted	Intracellular	Secreted
	dpm/μg cell protein (× 10^{-4})	*% of total*	*% of cellular protein*	*% of secreted protein*
5	3.1	1.1	0	0
7	3.2	1.4	0	0
9	6.4	3.6	0.3	1.8
12	4.0	2.6	0.4	3.1
14	4.1	3.3	0.4	4.9

* Trichloroacetic acid-insoluble radioactivity incorporated in cellular and secreted proteins was determined for BMM in 4-cm diam wells incubated with 25 μCi/ml [^{35}S]methionine for 2 h.

‡ Incorporation of radioactivity into ApoE was quantified by immunoprecipitation.

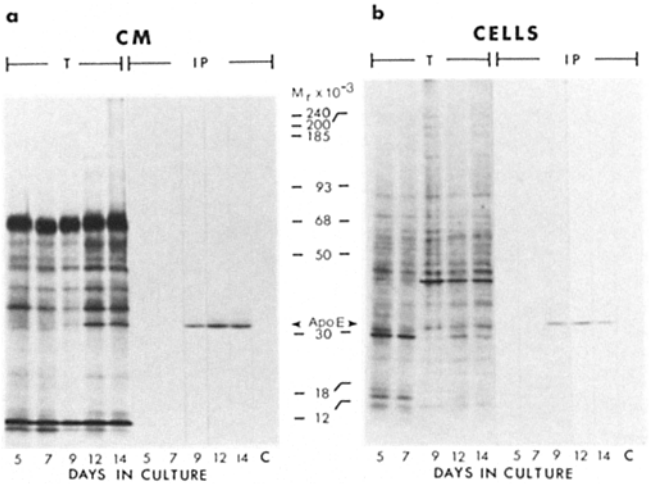

FIGURE 1 Immunoprecipitation of ApoE from (*a*) conditioned medium (*CM*), and (*b*) BMM cultured for 5–14 d in the presence of L-cell-conditioned medium as a source of CSF-1. *T*, total acid-precipitable proteins; aliquots containing $\sim 2 \times 10^4$ cpm of [^{35}S]methionine-labeled proteins were loaded in each well. *IP*, proteins immunoprecipitated with anti-ApoE IgG. The *IP* lanes marked *C* are controls showing proteins immunoprecipitated from the 14-d BMM with anti-bovine serum albumin IgG. M_r ($\times 10^{-3}$) markers and migration of ApoE from mouse very low-density lipoproteins are indicated.

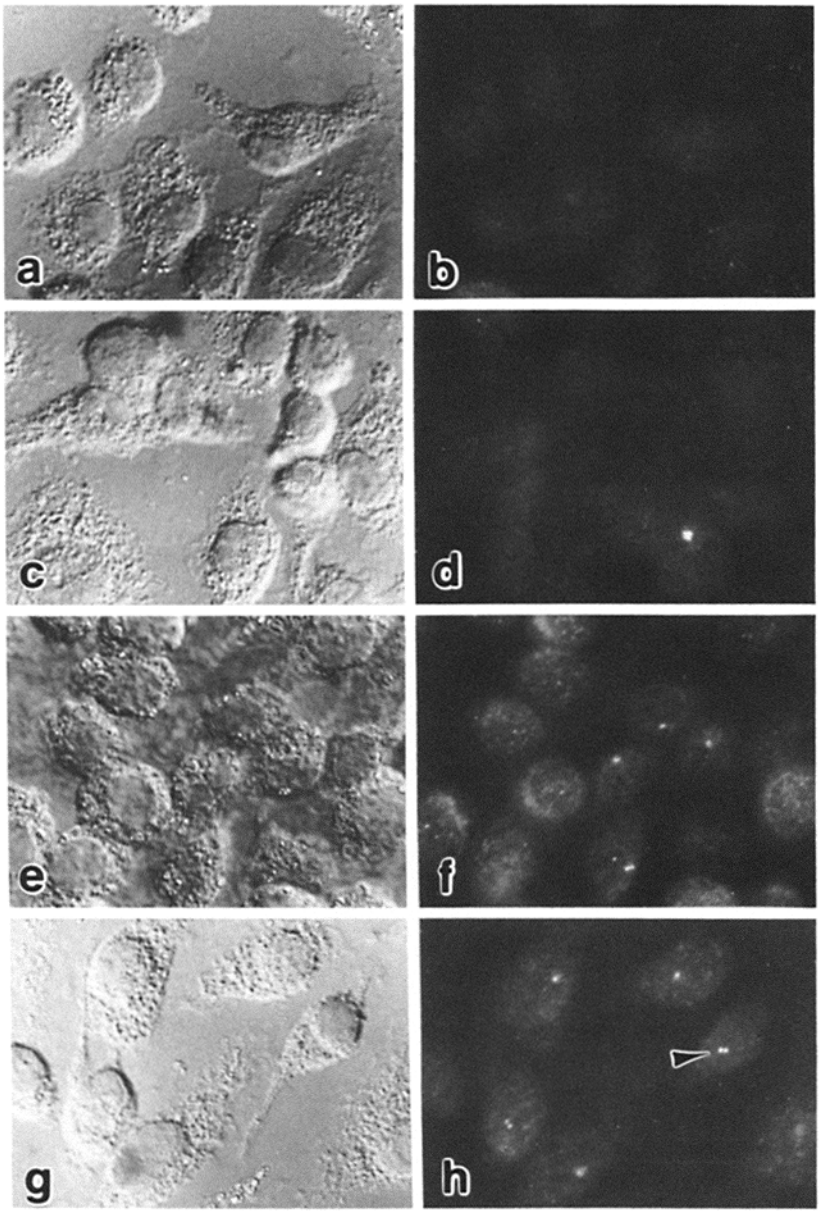

FIGURE 2 Immunofluorescent localization of ApoE in BMM cultured for (*a* and *b*) 5 d; (*c* and *d*) 7d; (*e* and *f*) 9d; (*g* and *h*) 12 d. Arrowhead in *h* indicates a macrophage containing intracellular ApoE. (*a*, *c*, *e*, and *g*) Phase-contrast microscopy; (*b*, *d*, *f*, and *h*) fluorescence microscopy. × 750.

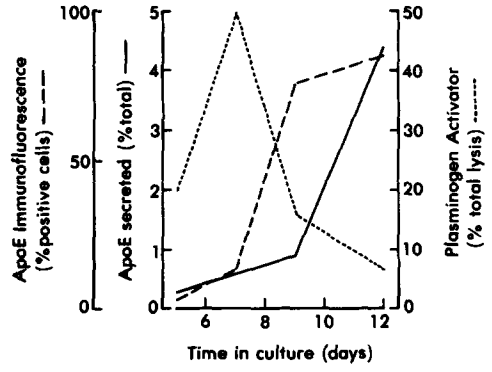

FIGURE 3 Time course of the appearance of cell-associated and secreted ApoE and secreted plasminogen activator in BMM cultures.

small density of fluorescence in the perinuclear area, increased in size between 9 and 12 d; total secretion also increased suggesting that ApoE secretion per cell increased as well, as has been shown for mature macrophages in culture (16–18). Once ApoE expression was initiated it was sustained at least 22 d (data not shown).

In previous work cholesterol-loaded resident macrophages have been shown to have an increased rate of secretion of ApoE (15, 18). When BMM at 5 and 7 d of culture were cholesterol-loaded, as judged by appearance of lipid droplets after uptake of acetylated low density lipoproteins (5 μg/ml), no ApoE was secreted suggesting that the developmental onset of ApoE secretion as a percentage of total secreted protein could not be accelerated. ApoE secreted, as a percentage of total secreted protein, by 12-d BMM was not altered by cholesterol loading.

Clonal Analysis of ApoE Expression

The expression of ApoE was studied in independently derived colonies of BMM plated at low density in culture to determine the heterogeneity of macrophages in this system. Although a few cells in some colonies expressed ApoE immunofluorescence by 7 d, most colonies were stained by 9 d and most cells appeared to become positive for ApoE syn-

chronously. By 12 d, >90% of the cells in all colonies were positive, and the amount of ApoE seen in individual cells was quite uniform (Figs. 2 and 3). Therefore, BMM of independent clonal origin all expressed ApoE.

Role of CSF-1 in Regulation of ApoE Expression by BMM and Primary Peritoneal Macrophages

In addition to inducing proliferation and differentiation of bone marrow cells, CSF-1 has been shown to induce secretion of plasminogen activator by BMM (4, 5). Because plasminogen activator secretion is increased in macrophages with reduced expression of ApoE (16), we studied the effect of L-cell-conditioned medium (the source of CSF-1) on ApoE expression in BMM and peritoneal macrophages. When 3-, 6-, and 13-d adherent BMM were washed and cultured for an additional 48 h in the absence of L-cell-conditioned medium, ApoE secretion was identical to that of BMM cultured with L-cell-conditioned medium (Table II). Therefore, the presence or withdrawal of the proliferative stimulus (L-cell-conditioned medium) did not affect expression of ApoE after onset of secretion.

When resident or thioglycollate-, $NaIO_4$- or BCG-elicited peritoneal macrophages were cultured in medium supplemented with fetal bovine serum, horse serum and L-cell conditioned medium for up to 21 d, all the elicited macrophages, but not resident macrophages, proliferated reaching a confluent monolayer by 21 d. When the secreted proteins of these cells were examined, the proliferating macrophages all secreted ApoE at high rates at 21 d. Without the L-cell conditioned medium the macrophages did not proliferate, but all expressed ApoE at this time. At 6 d, before the proliferating BMM initiated ApoE expression, the proliferating peritoneal macrophages secreted ApoE at high rates, as is shown in Table II for $NaIO_4$-elicited macrophages.

Comparison of Sequential Expression of ApoE with the Expression of Plasminogen Activator, Elastase, Fibronectin, and Cell Surface Markers by BMM

The onset of ApoE expression was next related to other markers of macrophage function. Lysozyme (M_r 14,000) secretion is initiated in very early BMM (7) and was constant by 5 d (Fig. 1). Initiation of ApoE synthesis and secretion correlated with a decrease in the expression of plasminogen activator, elastase, and fibrinolytic neutral proteinase (Fig. 3, Table III). Although activated macrophages express Ia antigens (10, 29, 30), BMM in culture did not have surface Ia at any time (Table III). Fibronectin (M_r 220,000) is not synthesized by monocytes, but is initiated as they become macrophages in vivo and in culture (16, 31). It is thus noteworthy that in ten different experiments, including that shown in Fig. 1, fibronectin secretion by BMM was not observed up to 14 d of culture or by elicited peritoneal macrophages proliferating in the presence of CSF-1 (data not shown). Other surface markers, including antigens F4/80, Mac-1, and nonspecific esterase, had a much earlier onset of expression than ApoE.

TABLE II

Expression of ApoE by Macrophages Cultured with or without CSF-1

Macrophage source	Initial culture with CSF-1	Final culture without CSF-1	ApoE secretion
	d	*d*	*% of secreted protein*
BMM	5	0	0
	3	2	0
	8	0	0.4
	6	2	0.4
	15	0	5.8
	13	2	6.1
$NaIO_4$-elicited	0	6	11.2
	6	0	9.6

Macrophages were cultured in the presence of L-cell-conditioned medium as a source of CSF-1, either for the complete culture period or for the initial culture period followed by culture without CSF-1 as indicated. ApoE secretion was determined by densitometry of fluorograms of [^{35}S]methionine-labeled secreted proteins.

Comparison of ApoE Expression by BMM, Peritoneal Macrophages in Various States of Activation, and Macrophage-like Cell Lines

The functional state of BMM as they initiated ApoE secretion was related to the functional states expressed by mature macrophages. The rate of ApoE secretion by BMM at 5 d in culture resembled that of BCG-activated macrophages, whereas the rate at 12 d resembled that of mature resident and thioglycollate-elicited macrophages (Table IV).

When BCG-activated macrophages were cholesterol-loaded, their rate of ApoE secretion, either on a per cell basis or as a percentage of total secreted protein, did not increase like that of BMM at 5 d. ApoE secretion was increased up to fivefold on a per cell basis by cholesterol-loading resident peritoneal macrophages, but remained constant as a percentage of total protein secretion like BMM at 12 d (data not shown).

Macrophage-like cell lines express phenotypes resembling various stages of differentiation of macrophages in vivo (21). Accordingly, we examined a variety of lines expressing a wide range of apparent differentiated phenotypes. None of the lines studied secreted significant quantities of ApoE (Table IV), even though P388D1 has a low rate of plasminogen activator secretion (20) and WEHI-3 and J774.2 have high rates (data not shown). Because ApoE expression is correlated with several important functional properties of macrophages (12), substituting these macrophage-like lines for macrophages may give rise to misleading biochemical and immunologic results.

DISCUSSION

Bone marrow proliferation in culture in response to CSF-1 (1-11) makes the various stages in the differentiation of mononuclear phagocytes accessible so that the developmental regulation of genes expressed by macrophages can be defined. In the present study we have shown that the onset of secretion of ApoE, a major gene product of mature macrophages in culture, occurs relatively late during differentiation, with expression beginning in most cells at 9 d. In bone marrow cultured without CSF-1, only the few cells with characteristics of mature macrophages expressed ApoE. We did not observe clonal heterogeneity for ApoE. Therefore, if the mononuclear phagocyte system consisted of more than one lineage, giving rise to the ApoE-nonsecreting macrophages found after BCG, pyran, or endotoxin activation (16, 17) and to the ApoE-secreting resident and thioglycollate-elicited macrophages (16, 17), this would have been reflected by differential expression

TABLE III

Sequential Expression of Functions during Macrophage Differentiation from Mouse Bone Marrow

Macrophage function	Resident peritoneal macrophages at 3 d	BMM, proliferating with CSF-1					Reference
		2–4 d	4–6 d	6–8 d	8–10 d	10–14 d	
ApoE, intracellular (% positive cells)	>95	<5	<5	<10	>70	>90	This study
Elastase secreted (U/mg cell protein/48 h)	12.2	ND*	45.3	41.2	4.1	ND	This study
Plasminogen activator secreted (U/mg cell protein/48 h)	72	ND	460	483	205	161	This study; 5, 6
Neutral proteinase secreted (U/mg cell protein/48 h)	ND	ND	338	331	18	44	This study
Fibronectin secreted (% total secretion)	5–10	<0.5	<0.5	<0.5	<0.5	<0.5	This study
Antigen Ia^k (% positive cells)	<3	ND	<1	<1	<1	<1	This study; 29
Antigen F4/80 (% positive cells)	100	ND	86	ND	98	100	3
Antigen Mac-1 (% positive cells)	100	ND	94	ND	100	99	3
Nonspecific esterase (% positive cells)	100	10	50	>90	100	100	3, 6

* ND, not determined.

TABLE IV

Comparison of ApoE Secretion by Macrophage-like Cell Lines and Macrophages in Various Stages of Maturation

Macrophage source	Time in culture	Apparent stage of mononuclear phagocyte maturation	ApoE secretion*
			% of total secreted protein
BMM	5 d	Immature macrophage	0.3
BMM	12 d	Mature macrophage	4.0
Resident peritoneal	2 h	Mature macrophage, nonproliferating	6.3
Thioglycollate-elicited	2 h	Mature, nonspecifically stimulated macrophage	6.7
BCG-activated	2 h	Mature, immunologically activated macrophage‡	0.4
MGI.D+, clone 11		Stem cell precursor‡	<0.5§
WEHI-3		Myelomonocyte‡	<1.0§
J774.2		Mature macrophage‡	<0.1§
P388D1		Mature macrophage‡	<0.05§
RAW 264.1		Mature macrophage‡	<0.5§
J774 × RAM-1		Mature macrophage‡	<1.6§

* ApoE secretion rate of peritoneal macrophages was determined by densitometry of fluorograms of secreted proteins labeled with [^{35}S]methionine for 2 h.

‡ Classification determined from properties described previously (19).

§ In these samples ApoE secretion was determined by measuring amount of M_r 33,000 protein co-migrating with ApoE, but was not confirmed by immunochemical methods.

of ApoE in clones or colonies arising from single precursors. Analysis of individual colonies for surface antigens has also provided no evidence for a clonal or lineage-dependent heterogeneity of macrophages (3, 6, 29). Thus, diversity of macrophages in tissues probably arises either from the modulation of expression of markers such as ApoE by the interaction of these cells with their extracellular milieu (16–18, 32), or from the recruitment of less mature cells from the bone marrow, particularly under conditions of stress.

Our studies also indicate that ApoE regulation in BMM is different from that in tissue macrophages. Whether or not peritoneal macrophages secreted ApoE immediately after being explanted into culture, secretion was initiated by 2–3 d of culture in medium containing serum and by 7 d ApoE was secreted in large quantities (16). In contrast, only after 9 d did BMM begin ApoE secretion, and then at relatively modest rates. Withdrawal of the source of proliferation-differentiation factor (CSF-1) from BMM did not prompt premature initiation of ApoE secretion, and CSF-1 did not suppress secretion of ApoE by mature peritoneal macrophages. Cholesterol loading of BMM did not induce ApoE secretion, whereas cholesterol-loaded mature macrophages modulated their ApoE secretion (18).

Initiation of ApoE secretion was correlated with a decrease in plasminogen activator secretion in both peritoneal macrophages (16) and BMM. However, the relationship of elastase secretion to ApoE secretion was different in mature macrophages and BMM. BCG-activated macrophages, which do not secrete ApoE, usually have very low elastase secretion (12, 16), whereas BMM secreted elastase at high rates until the onset of ApoE secretion. ApoE secretion is usually accompanied by secretion of fibronectin in mature macrophages in culture (16), but secretion of detectable fibronectin by BMM was not initiated in conjunction with the appearance of ApoE. A variety of macrophage-like cell lines expressing a range of macrophage phenotypes failed to secrete ApoE, indicating an uncoupling between ApoE expression and surface antigens and other markers (21–24) during acquisition of proliferative capacity. ApoE secretion is usually accompanied by secretion of fibronectin (M_r 220,000) in mature macrophages in culture (16), but secretion of detectable fibronectin by BMM was not initiated in conjunction with the appearance of ApoE.

Macrophage-derived ApoE may play a crucial role in controlling lipid metabolism and the immune response because ApoE has functions both in the transport of lipoproteins (33) and in immunoregulation (16, 34, 35). The immature BMM and immunologically activated macrophages (16, 17) are the physiologic counterparts of the monensin-treated macrophages that have inhibited secretion of ApoE (36). Because macrophages that do not secrete ApoE still secrete cholesterol (36), the "reverse" cholesterol transport mechanisms involving ApoE may be disrupted in sites of inflammation where

immature or activated macrophages may be recruited. The decreased local production of ApoE under these conditions may also serve to stimulate the immune functions that are suppressed by ApoE (34, 35). The potential regulation of secretion of ApoE by three distinct regulatory systems, one related to differentiation of mononuclear phagocytes (this paper), a second to immunologic regulation (16), and a third to cholesterol metabolism (15, 18), complicates the analysis of the contribution of macrophages to ApoE production in vivo. As molecular probes are developed, ApoE secretion will provide an interesting and accessible system for the investigation of a gene regulated developmentally and environmentally in macrophages.

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